The EMBO Journal vol.15 no.23 pp.6584–6594, 1996

The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth

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Phosphoinositide 3-kinases (PI3Ks) have been identified in an evolutionarily diverse range of organisms, including mammals, *Drosophila*, yeast, plants and *Dictyostelium*. They are activated by a multitude of extracellular signals and implicated in mitogenesis, differentiation and cell survival, as well as in the control of the cytoskeleton and cell shape. Here we describe the molecular and functional analysis of *Drosophila* p110 (Dp110). A full-length Dp110 cDNA was isolated and found to encode a protein homologous throughout its length to the class I mammalian PI3Ks p110α and p110β. Overexpression of Dp110 in wing or eye imaginal discs resulted in flies with enlarged wings or eyes respectively. In contrast, overexpression of Dp110 containing a mutation predicted to result in the loss of catalytic activity resulted in smaller wings and eyes. The alterations in wing size result from changes in both cell size and cell number, whereas in the eye only differences in cell size were detected. These data imply a role for Dp110 in growth control during *Drosophila* development and have implications for the function of class I PI3Ks in other organisms.

Keywords: cell number/cell size/Dp110/growth/phosphoinositide 3-kinases

Introduction

Biochemical analyses suggest that all eukaryotic cells produce inositol lipids phosphorylated at the D3 position of the inositol ring, yet our current understanding of the role of these lipids *in vivo* is poor. These phospholipids are generated by phosphoinositide 3-kinases (PI3Ks), which catalyse the phosphorylation of phosphatidylinositol (PtdIns), PtdIns (4)P and PtdIns (4,5)P_2, thereby generating PtdIns (3)P, PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 respectively. As yet, no phospholipase activity able to catabolize these D3-phosphoinositides has been described (Lips *et al.*, 1989; Serunian *et al.*, 1989), so it has been proposed that they themselves act as second messengers. The cloning and characterization of PI3Ks from mammals, *Drosophila*, yeast, soybean and *Dictyostelium* has enabled subdivision of the PI3K superfamily into three classes. These classes are distinguishable not only on the basis of primary structure, but also by their *in vitro* substrate specificity and their likely mechanism of regulation and function *in vivo* (MacDougall *et al.*, 1995; Zvelebil *et al.*, 1996).

Class I PI3Ks are regulated by cell surface receptors and phosphorylate PtdIns, PtdIns (4)P and PtdIns (4,5)P_2 *in vitro*, though PtdIns (4)P and PtdIns (4,5)P_2 are thought to be their major substrates *in vivo* (Stephens *et al.*, 1993). They include those enzymes which most closely resemble the prototypical p110 catalytic subunit (Hiles *et al.*, 1992) and which associate with a regulatory adaptor subunit [e.g. p85α, p85β (Otsu *et al.*, 1991) and p55PIK (Pons *et al.*, 1995)]. The regulatory subunits contain SH2 domains that bind to specific phosphotyrosine residues and recruit p110/p85 heterodimers to activated receptor tyrosine kinases (RTKs), thereby facilitating their activation. These class I PI3Ks may also be regulated by additional mechanisms. For example, p85 can interact with other regulatory proteins via its SH3 domain, Bcr homology domain and proline-rich sequences, whereas p110 can associate with and be activated by the GTP-bound form of the small GTP binding protein Ras (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994, 1996). More recently, class I PI3Ks that are activated by heterotrimeric G proteins and associate with novel regulatory subunits have also been described (Stephens *et al.*, 1994; Stoyanov *et al.*, 1995).

Class II PI3Ks contain regions homologous to the catalytic core of the class I enzymes plus an extended N-terminus and a C-terminal C2 domain, both of unknown function (MacDougall *et al.*, 1995; Molz *et al.*, 1996; Virbasius *et al.*, 1996). *In vitro* they phosphorylate PtdIns and PtdIns (4)P but not PtdIns (4,5)P_2. Thus, both the mode of regulation by upstream signals and the downstream targets of class II PI3Ks may differ from those in class I.

Members of PI3K class III include yeast Vps34p (Schu *et al.*, 1993) and human (Volinia *et al.*, 1995), *Drosophila* (MacDougall *et al.*, 1995; Linassier *et al.*, 1996) and *Dictyostelium* (Zhou *et al.*, 1995) homologues. *In vitro* these enzymes can only phosphorylate PtdIns, generating PtdIns (3)P, which is present at steady-state levels in resting and in stimulated cells. Genetic studies indicate a 'housekeeping' role for yeast Vps34p in the sorting and trafficking of newly synthesized proteins, so class III PI3Ks may serve an analogous role in higher eukaryotes.

Thus far, the analysis of PI3K function has focused on the role played by class I p110/p85 PI3Ks following their activation by RTKs. Autophosphorylated RTKs recruit multiple signalling molecules to membrane-bound complexes via the interaction of phosphotyrosines with SH2 and PTB domains and thereby activate a number of intracellular signalling pathways (Pawson, 1995). The best characterized of these, the Ras/MAP kinase pathway,

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seems on its own to be sufficient to mediate many of the changes normally associated with the activation of specific RTKs. In contrast, the purpose of recruiting signalling molecules such as PI3K, phospholipase Cγ (PLCγ) and p120 RasGAP is less clear. PI3K function in mammalian cells has been probed using RTKs mutated at their PI3K binding sites, PI3K inhibitors, neutralizing antibodies and dominant-negative versions of p85. These experiments implicate PI3Ks in numerous cellular processes, including mitogenesis, cell survival, differentiation, chemotaxis, receptor trafficking and reorganization of the actin cytoskeleton (Vanhaesebroeck *et al.*, 1996). However, we still know very little about how exactly PI3Ks are involved in these diverse physiological processes. Possible PI3K targets include the small GTP binding protein Rac (Wennstrom *et al.*, 1994; Hawkins *et al.*, 1995) and the serine/threonine kinases c-Akt (also known as RAC and PKB) (Burgering and Coffer, 1995; Franke *et al.*, 1995), $p70^{S6kinase}$ ($p70^{S6K}$) (Cheatham *et al.*, 1994; Chung *et al.*, 1994) and certain PKC isoforms (Nakanishi *et al.*, 1993; Toker *et al.*, 1994; Palmer *et al.*, 1995).

To gain more insight into the function of PI3Ks and D3-phosphorylated lipids, we have initiated a study of PI3Ks in *Drosophila*. Degenerate PCR revealed that *Drosophila* possesses genes encoding at least one enzyme from each PI3K class (MacDougall *et al.*, 1995). Here we present the molecular and biochemical characterization of *Drosophila* p110. Sequencing of a full-length cDNA encoding Dp110 revealed that it is homologous throughout its length to the class I mammalian PI3Ks p110α and p110β and the recently identified p110δ (B.Vanhaesebroeck *et al.*, in preparation). We show that Dp110 associates via a p85-like adaptor protein, p60, with the same phosphotyrosine motifs as mammalian p110/p85 and that the Dp110/p60 complex possesses phosphoinositide 3-kinase activity *in vitro*. We have used the UAS-GAL4 system (Brand and Perrimon, 1993) to examine the effect of overexpressing wild-type, membrane-targeted and catalytically inactive Dp110. Wing and eye imaginal discs from third instar larvae and the adult structures that they give rise to are increased in size by expression of wild-type or membrane-targeted Dp110 and decreased in size by expression of catalytically inactive Dp110. These changes in size are mediated via effects on both cell size and cell number. The induction of these phenotypes by ectopically expressed Dp110 strongly suggests that endogenous Dp110 is involved in the control of cell growth during imaginal disc development, presumably following RTK activation.

Results

Isolation of cDNAs encoding the Drosophila PI3K, Dp110

Degenerate PCR with primers based on conserved regions in the kinase domains of mammalian p110α and yeast Vps34p was used to isolate a unique fragment corresponding to part of the gene encoding Dp110 (MacDougall *et al.*, 1995). This ~400 bp fragment was used as a probe to isolate Dp110 cDNAs. The longest cDNA isolated contained 2639 bp of coding sequence and ~900 bp of 3′-untranslated sequence, but lacked a start codon. 5′ rapid amplification of cDNA ends (RACE) PCR from the same library generated a fragment with an addititonal 628 bp of coding sequence and 295 bp of 5′ untranslated sequence containing several in-frame stop codons (see Materials and methods).

The Dp110 open reading frame encodes a 1088 amino acid protein (Figure 1A) with a predicted molecular mass of 127 kDa and with substantial sequence homology throughout its length to mammalian p110α, p110β and p110δ (38, 39 and 40% overall identity with the human isoforms respectively). As is illustrated by the dendrogam shown in Figure 1B, Dp110 is a class I PI3K. While the existence of another *Drosophila* class I PI3K cannot be ruled out, we have detected no such genes by degenerate PCR, screening cDNA and genomic DNA libraries, or *in situ* hybridization to polytene chromosomes. We have recently revised the cytological position of Dp110 to 92E12-13.

Based on sequence homology, particularly with other class I PI3Ks, Dp110 can be divided up into domains of different predicted function (Figure 1C; Zvelebil *et al.*, 1996). The most conserved regions, found at the C-terminus, are the catalytic core (HR1) and PIK domain (HR2), a domain that is also found in PI4-kinases. The N-terminus contains regions known to be both necessary and sufficient for mammalian p110α to associate with the regulatory subunit p85 (Dhand *et al.*, 1994a) and Ras (Rodriguez-Viciana *et al.*, 1996). The central portion of the protein contains two further PI3K homology regions, HR3, which is of unknown function, and a stretch of basic amino acids followed by a short leucine zipper that is reminiscent of the dimerization domains of bZIP transcription factors and which therefore might play a role in intra- or intermolecular interactions (B.Vanhaesebroeck, M.J.Welham, K.Kotani, R.Stein, P.H.Warne, M.J.Zvelebil, K.Higashi, S.Volinia, J.Downward and M.D.Waterfield, in preparation)

Dp110 exists as a heterodimer with a phosphotyrosine binding adaptor protein

We next investigated whether Dp110, like its mammalian homologues, exists as a heterodimer able to bind to specific phosphotyrosine residues. We hypothesized that a Dp110 adaptor protein might recognize the same pYXXM motif as its mammalian counterparts and consequently performed affinity purifications with a phosphopeptide containing two pYXXM motifs found in the human PDGFβ receptor. pYXXM binding proteins were purified from *Drosophila* Schneider S2 cells and, as a positive control, human U937 cells, then the proteins resolved by SDS–PAGE and revealed by silver staining. The two subunits of the human p85/p110 heterodimer were purified from human U937 cells (Figure 2A, lane 2), whereas three predominant proteins with approximate molecular masses of 145, 120 and 60 kDa were reproducibly isolated from the S2 cell lysates (Figure 2A, lane 1). Peptide microsequencing (data not shown) and immunoblotting with an antibody raised against a Dp110 peptide (Figure 2A, lanes 3 and 4) demonstrated that p120 was Dp110. p145 was generally seen in substoichiometric amounts and, unlike p120 and p60, could be completely removed by washing in 2 M NaCl. Peptide microsequencing identified p145 as *Drosophila* PLCγ (Emori *et al.*, 1994). The SH2 domains of mammalian PLCγ have less specific binding sites than those of p85 and human PLCγ can also bind to

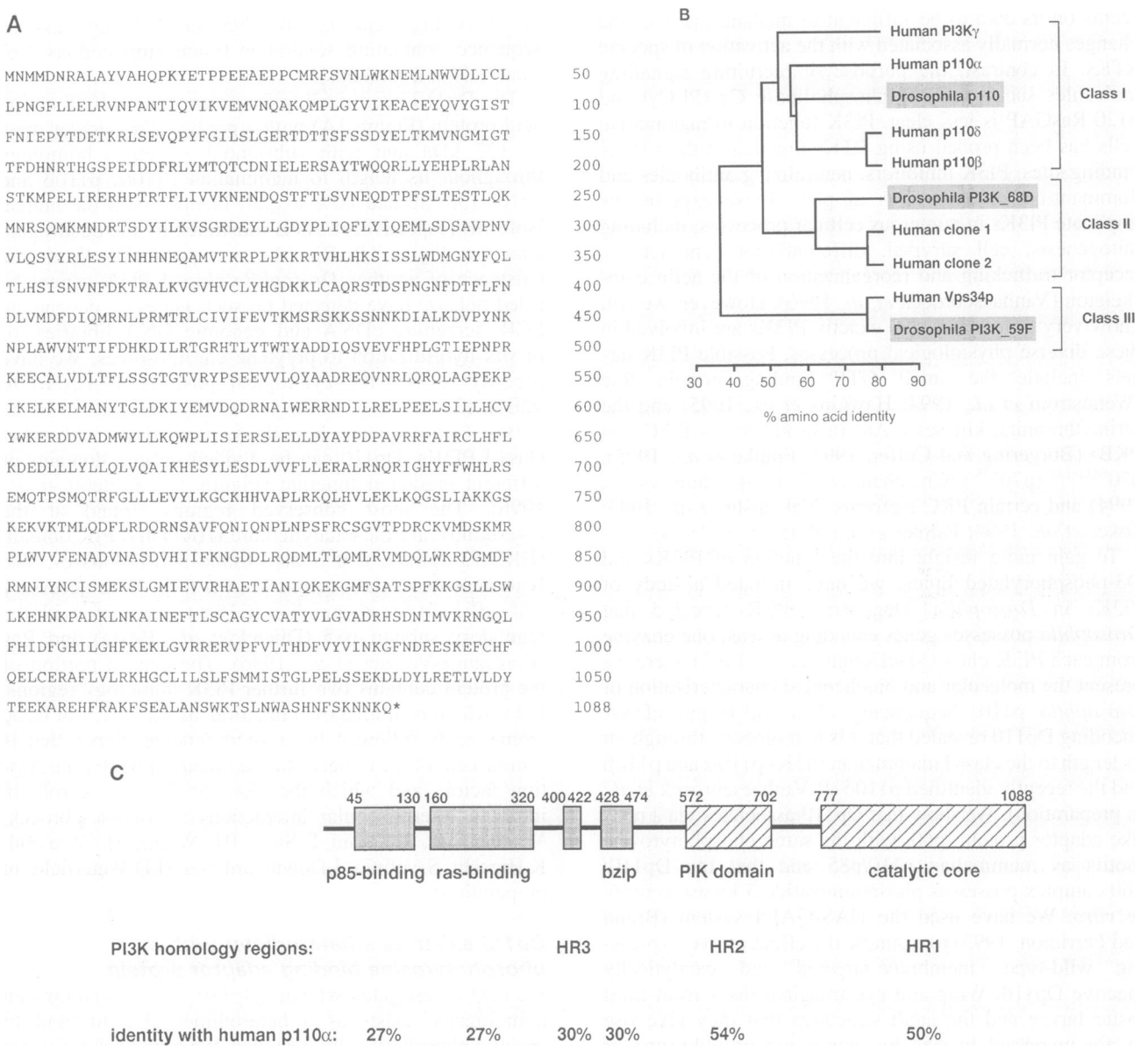

Fig. 1. Molecular characterization of Dp110. (**A**) Predicted amino acid sequence of Dp110. (**B**) Dendrogram showing phylogenetic relationship between the catalytic domains of Dp110 and selected human PI3Ks, including p110δ and human clones 1 and 2, class II PI3Ks currently being characterized in our laboratory (S.Volinia, R.Vanhaesebroeck, F.Pagès, J.Domin and M.D.Waterfield). The dendrogram was generated using the PILEUP program. (**C**) Schematic diagram of the primary structure of Dp110 showing regions conserved in mammalian p110s and per cent identity of each region with that of p110α.

pYXXM motifs (I.Gout, personal communication). Peptide microsequencing of p60 suggested that it might represent an SH2 domain-containing adaptor protein for Dp110. Subsequent degenerate PCRs and cDNA cloning have confirmed that p60 is an adaptor protein containing two SH2 domains separated by a region with significant sequence homology to the p110 binding site of mammalian p85s (D.Weinkove, S.J.Leevers, M.D.Waterfield, unpublished data).

The Dp110/p60 complex possesses lipid kinase activity

Although the high degree of sequence conservation between Dp110 and mammalian p110s in HR1 and HR2 suggests that Dp110 is a PI3K, we sought to demonstrate this biochemically. Assays were performed using the affinity-purified Dp110/p60 complex and substrates presented in mixed lipid vesicles. In these assays, Dp110/p60 catalysed the *in vitro* phosphorylation of PtdIns, PtdIns (4,5)P_2 and, to a lesser extent, PtdIns (4)P (Figure 2B). Anion exchange high pressure liquid chromatography confirmed that the PtdIns monophosphate generated by PtdIns phosphorylation was phosphorylated at the D3 position of the inositol ring (data not shown).

Analysis of Dp110 function by ectopic expression

We have used a reverse genetic approach to initiate a study of PI3Ks in *Drosophila*. To gain insight into the function of Dp110, we used the UAS-GAL4 system to express Dp110 ectopically at different stages of *Drosophila* development (Brand and Perrimon, 1993). cDNAs encoding wild-type Dp110 (WT-Dp110), membrane-targeted

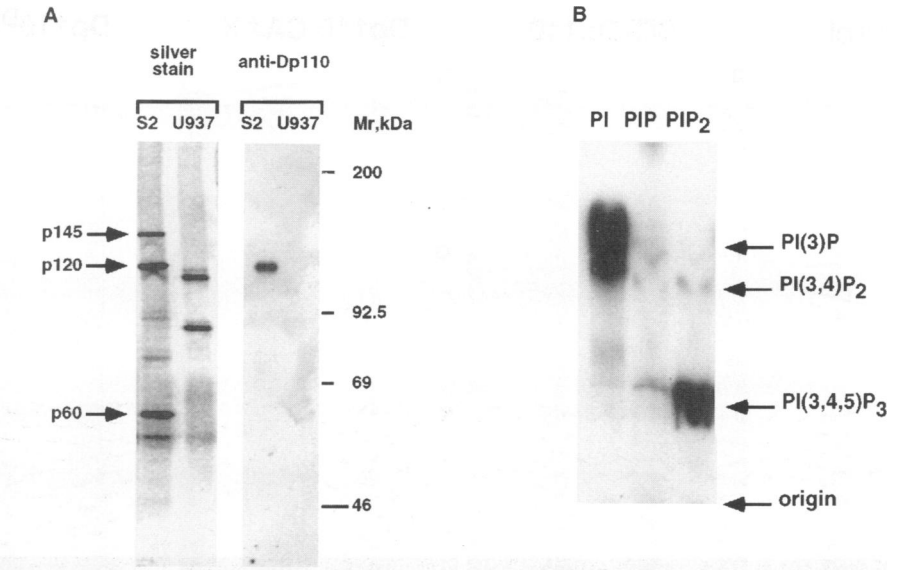

Fig. 2. Biochemical characterization of Dp110. (**A**) Affinity purification of Dp110 on pYXXM phosphopeptides. pYXXM beads were incubated with lysates from *Drosophila* S2 cells or human U937 cells, washed and bound proteins resolved by SDS–PAGE and revealed by silver staining (lanes 1 and 2) or by Western blotting with anti-Dp110 (lanes 3 and 4). p145, p120 and p60 were not detected when beads coupled in the absence of phosphopeptide were used as a control (data not shown). (**B**) Lipid kinase activity of Dp110/p60. Assays of Dp110/p60 PI3K activity were performed as described using PtdIns (PI), PtdIns (4)P (PIP) or PtdIns (4,5)P_2 (PIP_2) as substrates. No PI3K activity was detected on control beads lacking peptide (data not shown).

Dp110 (Dp110-CAAX) and Dp110 with a mutation in the putative ATP binding site ($Dp110^{D954A}$) were cloned into P element expression vectors under the control of yeast GAL4 upstream activating sequences (UAS). We hypothesized that membrane-targeted Dp110 would have better access to lipid substrates and therefore be more active than the wild-type protein *in vivo*, as has recently been shown for mammalian p110 (Klippel *et al.*, 1996). A membrane-targeted version of Dp110 was generated by the addition of the 20 C-terminal amino acids of mammalian Ras, which are sufficient for the post-translational modification and membrane localization of heterologous proteins (Leevers *et al.*, 1994). Conversely, overexpressed, catalytically inactive $Dp110^{D954A}$ should compete with endogenous Dp110 for binding sites on interacting molecules such as p60 or Ras, thereby having an inhibitory or 'dominant-negative' effect on the function of endogenous Dp110 *in vivo*. P element-mediated germline transformation was used to generate several independent transgenic lines carrying each construct, so that the possibility of insertion site-mediated effects could be ruled out.

Three independent transformants for each construct were crossed with lines expressing GAL4 in specific patterns and the progeny analysed. We looked for the generation of opposing phenotypes by the expression of wild-type or membrane-targeted Dp110 and the putative dominant-negative $Dp110^{D954A}$. Such phenotypes were generated by the expression of Dp110 in imaginal discs, sheaths of epithelial cells which are set aside during embryogenesis, which expand and differentiate during larval growth and which ultimately give rise to the structures that make up the adult fly (Cohen, 1993). *In situ* hybridization with Dp110 mRNA probes confirmed that Dp110 mRNA is normally expressed in the wing and eye imaginal discs (data not shown). This localization is consistent with the assumption that the Dp110 transgenes induce the phenotypes described by altering signalling pathways in which endogenous Dp110 normally functions.

Ectopic Dp110 expression affects wing growth

The adult wing consists of two layers of ectodermal cells (dorsal and ventral) that secrete cuticular 'structures' (wing hairs and veins) and contain sensory organs (campaniform sensilla on the wing surface and sensory bristles along the anterior wing margin). We examined the effect on wing development of Dp110 expression during larval stages, when the wing imaginal disc is a monolayer epithelium divided by anterior/posterior (A/P) and dorsal/ventral (D/V) compartment boundaries. The expression of WT-Dp110 or Dp110-CAAX in different regions of the wing imaginal disc resulted in expansion of the corresponding regions of the adult wing blade. Conversely, $Dp110^{D954A}$ expression reduced the size of these regions. For example, expression of WT-Dp110 or Dp110-CAAX in the prospective dorsal surface of the wing blade resulted in wings that were bigger than wild-type (Figure 3B and C) and curved downwards. In contrast, $Dp110^{D954A}$ wings were smaller (Figure 3D) and curved upwards. Similarly, when Dp110 was expressed along, and immediately anterior to, the A/P boundary of the wing disc, the corresponding region of the adult wing (visualized by assessing the distance between longitudinal veins III and IV) expanded or contracted (Figure 3E–H). Interestingly, the proximal to distal alignment of the non-sensory wing hairs present on the surface of the wing blade was often disrupted by the expression of WT-Dp110 or Dp110-CAAX (Figure 3K).

To further examine the effect of Dp110 on wing growth, we looked at wing discs from third instar larvae in which GAL4 was driving the co-expression of nuclear β-galactosidase and different forms of Dp110 at high levels in the dorsal wing pouch and at lower levels in the

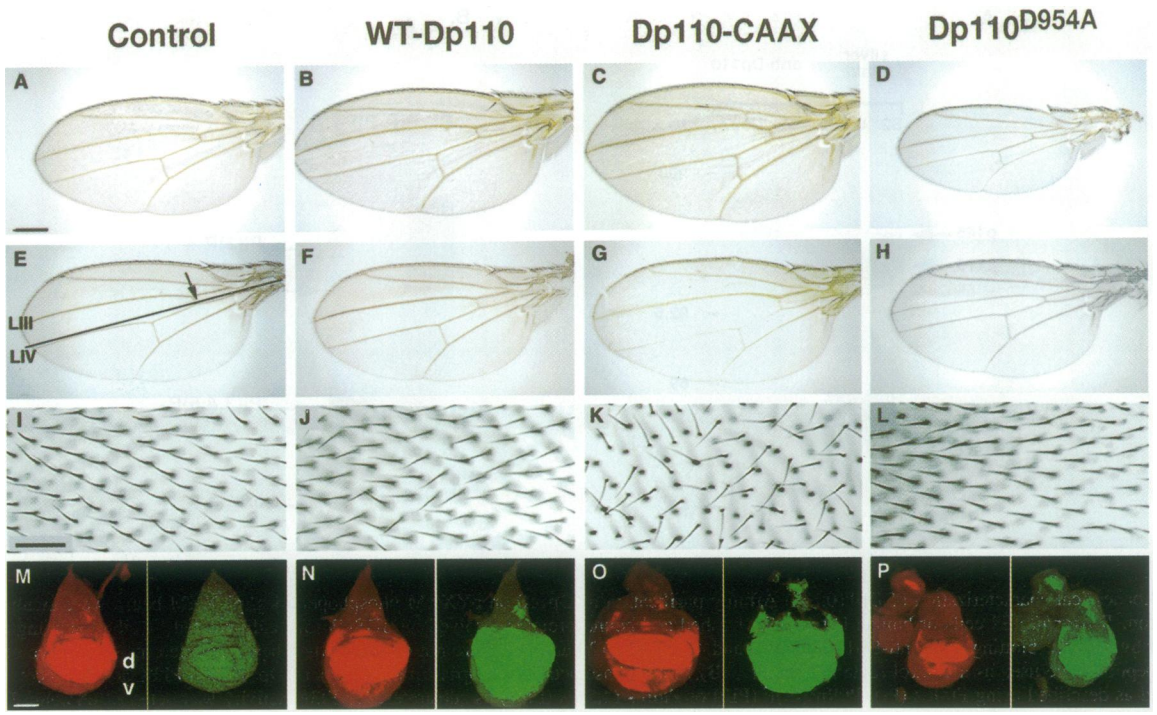

Fig. 3. Wild-type and membrane-targeted Dp110 expression promote wing growth, whereas catalytically inactive Dp110 inhibits wing growth. (**A–D**) Wings of flies expressing Dp110 and GAL4 in the dorsal wing pouch. (A–H) Bar 250 μm. (**E–L**) Wings of flies expressing Dp110 and GAL4 along and just anterior to the anterior/posterior (A/P) boundary. (E) The A/P boundary is indicated by a line and the anterior cross vein by an arrow. Longitudinal veins III and IV are also shown. (I–L) Distribution and orientation of wing hairs just distal to the anterior cross vein; proximal is left. Bar 25 μm. (**M–P**) Confocal images of wing imaginal discs from third instar larvae expressing Dp110, β-galactosidase (β-gal) and GAL4 in the dorsal wing pouch. β-galactosidase expression is shown in red and epitope-tagged Dp110 in green; the dorsal (d) and ventral (v) wing pouches are indicated; anterior is left, dorsal is up. Bar 100 μm. Similar results were obtained with different Dp110 P element insertion lines. Flies were of the following genotypes: (A) *GAL4/+*, (B) *GAL4/+*; *UAS-WT-Dp110/+*, (C) *GAL4/+*; *UAS-Dp110-CAAX/+*, (D) *GAL4/+*; *UAS-Dp110^{D954A}/+*, (E and I) *dpp-disc-GAL4/+*, (F and J) *UAS-WT-Dp110*; *dpp-disc-GAL4/+*, (G and K) *UAS-Dp110-CAAX*; *dpp-disc-GAL4/+*, (H and L) *UAS-Dp110^{D954A}*; *dpp-disc-GAL4/+*, (M) *GAL4/+*; *UAS-β-gal/+* (N) *GAL4/+*; *UAS-WT-Dp110/+*; *UAS-β-gal/+* (O) *GAL4/+*; *UAS-Dp110-CAAX/+*; *UAS-β-gal/+* (P) *GAL4/+*; *UAS-Dp110^{D954A}/+*; *UAS-β-gal/+*.

ventral wing pouch (Figure 3M–P). The region of GAL4 expression was revealed by immunostaining for β-galactosidase expression (in red, left-hand panel), and ectopic Dp110 expression was detected by a monoclonal antibody directed against a myc peptide epitope engineered onto the N-terminus of the Dp110 expression constructs (in green, right-hand panel). Consistent with the effects observed in adult wings, the GAL4 expression domain was expanded by WT-Dp110 and Dp110-CAAX and contracted by Dp110^{D954A}. Similar differences were observed in discs expressing Dp110 along the A/P boundary, even in the regions that give rise to non-wing blade adult structures, such as the notum and hinge (data not shown). Significantly, the same effects were also observed in leg and haltere discs expressing Dp110 along the A/P boundary, indicating that the effect of Dp110 on the growth of imaginal discs is not specific to wing discs.

We next examined whether the observed differences in wing size resulted from alterations in cell size or cell number. The number of cells per unit area was assessed by counting wing hairs, single apical extensions found on the surface of each wing blade cell. Wings generated by transgene expression along the A/P boundary were analysed by examining an area of fixed size on the the wing blade (Table I, columns 1–4), and by looking on the wing margin between veins III and IV (Table I, columns 5–7). Surprisingly, we found that Dp110 and Dp110-CAAX expression increased both the overall number of cells and their size. In contrast, Dp110^{D954A} decreased both cell size and cell number.

Ectopic Dp110 expression affects eye growth and organization

To further analyse the role that Dp110 might play in the growth of imaginal discs, we examined the effects of ectopic expression during eye development. The *Drosophila* compound eye is a repetitive and highly organized structure generated by the stepwise recruitment of cells to ommatidial clusters behind an indentation in the eye imaginal disc known as the morphogenetic furrow. These clusters grow and differentiate during larval and pupal development and ultimately give rise to the adult retina (Wolff and Ready, 1993). The Dp110 transgenes were expressed in cells posterior to the morphogenetic furrow during late larval and early pupal development using a GMR-GAL4 line (see Materials and methods).

The expression of wild-type (Figure 4B) or membrane-targetted Dp110 (data not shown) generated enlarged and bulging, roughened eyes with fused ommatidia and misplaced or duplicated bristles, whereas Dp110^{D954A} eyes were smaller and flatter (Figure 4C). The hexagonal lenses or facets that form the surface of each ommatidium were larger (WT-Dp110) or smaller (Dp110^{D954A}). The small eyes contained the wild-type number of facets (775 ± 9, n = 4 for control eyes; 777 ± 8, n = 4 for Dp110^{D954A} eyes), whereas the enlarged eyes actually contained fewer

Table I. Effect of Dp110 transgene expression on wing cell number and size[a]

Genotype	1 Area bound by LIII, ACV, LIV and wing margin (μm^2)[b]	2 Cell density (cells/μm^2)[c]	3 Approximate No. of cells in area bound by LIII, ACV, LIV and wing margin[d]	4 Area covered per cell (μm)[e]	5 Length of margin between LIII and LIV (μm)[b]	6 No. of margin cells between LIII and LIV[f]	7 Wing margin cell diameter (μm)[g]
dpp-disc-GAL4/+[h]	2.5×10^5	$6.2\times10^{-3} \pm 0.2\times10^{-3}$	1.5×10^3	161	203 ± 2	19	10.7
UAS-WT-Dp110; dpp-disc-GAL4/+	2.6×10^5	$6.2\times10^{-3} \pm 0.2\times10^{-3}$	1.6×10^3	161	235 ± 2	21	11.2
UAS-Dp110-CAAX; dpp-disc-GAL4/+	3.7×10^5	$5.4\times10^{-3} \pm 0.2\times10^{-3}$	2.0×10^3	185	265±14	24	11.4
UAS-Dp110^{D954A}; dpp-disc-GAL4/+	1.6×10^5	$8.3\times10^{-3} \pm 0.1\times10^{-3}$	1.3×10^3	120	151 ± 5	17	8.9

[a]Four wings of each genotype were analysed, standard deviations are shown where they are within the number of significant figures given.
[b]Measured using NIH Image 1.60.
[c]Assessed by counting number of wing hairs on one wing surface in a 13 000 μm^2 area just distal to the ACV.
[d]Generated by multiplying the values in column 1 by those in column 2.
[e]Reciprocal of column 2.
[f]Assessed by counting the number of hairs along the margin of one wing surface between veins LIII and LIV.
[g]Generated by dividing the values in column 5 by those in column 6.
[h]Dp110 transgene expression was directed along and immediatedly anterior to the A/P boundary by a dpp-disc-GAL4 transgene (Staehling-Hampton *et al.*, 1994).

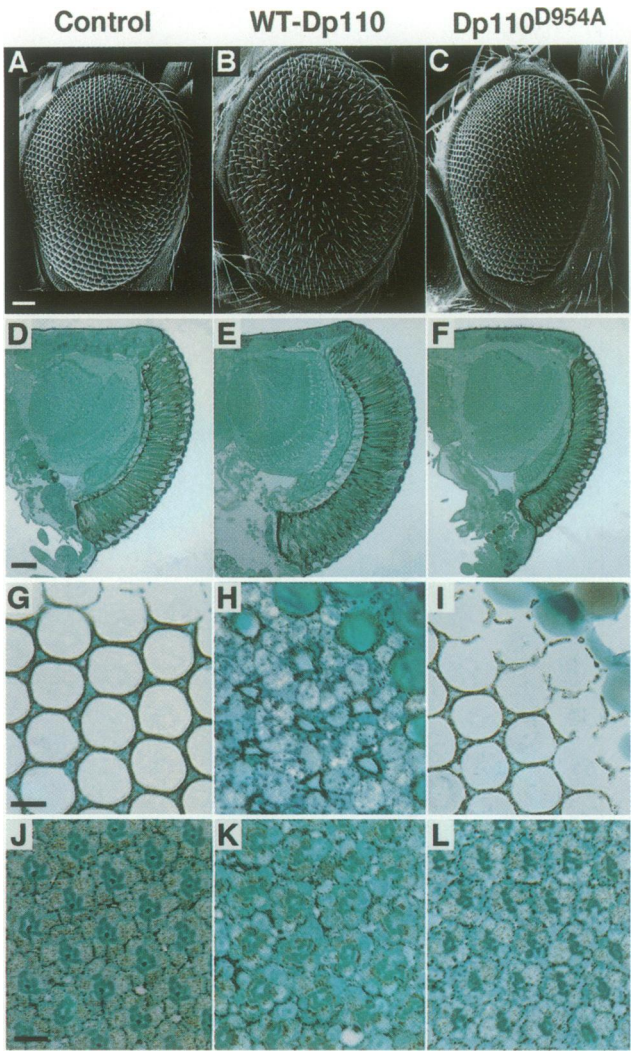

Fig. 4. Overexpression of WT-Dp110 generates large rough eyes, whereas overexpression of Dp110^{D954A} generates smaller eyes. (**A–C**) Scanning electron micrographs of adult eyes; anterior is left. Bar 50 μm. (**D–F**) Radial sections through adult eyes; dorsal surface of head is up. Bar 50 μm. (**G–I**) Tangential sections through adult eyes; sections are from just below the lens; the space normally occupied by the pseudocone and surrounded by pigment and bristle cells (blue and grey) can be seen in the wild-type eye (G). Bar 10 μm. (**J–L**) Tangential sections through adult eyes; the photoreceptor rhabdomeres can be seen at the centre of each ommatidium (dark blue ovals). Bar 10 μm. Similar results were obtained with different Dp110 P element insertion lines. Flies were of the following genotypes: (A, D, G and J) *GMR-GAL4/+*, (B, E, H and K) *GMR-GAL4, UAS-WT-Dp110/UAS-WT-Dp110*, (C, F, I and L) *GMR-GAL4, UAS-Dp110^{D954A}/+; UAS-Dp110^{D954A}*.

ommatidia (710 ± 5, n = 3 for WT-Dp110). To investigate the internal changes resulting in these differences, radial and tangential sections through the eyes were examined (Figure 4D–L). The radial sections reflected differences seen from the outside; the adult retina was increased (Figure 4E) or decreased (Figure 4F) in both size and thickness. In addition, in WT-Dp110 eyes the apical region above the photoreceptor rhabdomeres and immediately behind the lens was filled with cells. This was particularly evident in apical tangential section, where the space occupied by the secreted pseudocone in control eyes (Figure 4G) contained many swollen cell bodies in WT-Dp110 eyes (Figure 4H). In more basal transverse sections the normally regular array of photoreceptors was also disrupted by WT-Dp110 expression. The rhabdomeres appeared twisted and were disorganized with respect to each other and neighbouring ommatidia, and the number of photoreceptor rhabdomeres was often reduced (Figure 4K). In Dp110^{D954A} eyes the lattice was also disrupted, though to a lesser degree, and the orientation of photoreceptors relative to those in neighbouring ommatidia was at least partially maintained (Figure 4L).

These adult eye phenotypes indicated that the Dp110 transgenes can also modulate growth during eye development, so we looked more closely for differences in cell size and/or cell number by examining confocal images of fluorescently labelled larval and pupal discs. The process of photoreceptor determination, as judged by the pattern of expression of the neuronal marker Elav (Robinow and White, 1991), was unaffected by WT-Dp110 and Dp110^{D954A} expression (Figure 5A–C). Interestingly though, the nuclei of WT-Dp110 discs were more widely spaced (compare Figure 5A and B). Similarly, the distance between the centres of the developing ommatidial clusters, revealed by immunostaining larval (Figure 5D–F, left hand panels) and pupal (Figure 5D–F, right hand panels) discs with anti-Armadillo, was affected by Dp110 transgene expression. Armadillo, the *Drosophila* homologue of vertebrate β-catenin, localizes to the adherens junctions (Peifer *et al.*, 1994) just below the apical membranes of the developing photoreceptors, where the cells are constricted and in close contact. The apical clustering of the photoreceptor membranes was disrupted in Dp110^{D954A} pupal discs, where a 'ring' of Armadillo staining that persisted more basally than in control discs was seen (Figure 5F, right hand panel).

Immunostaining of the membranes of pupal disc cells with anti-α-Spectrin indicated that the Dp110 transgenes affected the size and not the number of cells in the eye disc (Figure 5G–I). The pigment, cone and photoreceptor cell bodies were swollen in the WT-Dp110 discs and smaller in the Dp110^{D954A} discs. Notably, in spite of these differences in cell size, the specific arrangement of the different cell types within each ommatidial cluster was undisturbed. Consistent with this, cell proliferation (examined by bromodeoxyuridine incorporation) and apoptosis (examined by staining with acridine orange) were not detectably affected in the developing eye discs (data not shown). Presumably the roughness and degeneration of the ommatidial pattern seen in adult WT-Dp110 eyes must arise during the late pupal stages of eye development.

Discussion

Determination of the predicted amino acid sequence of the *Drosophila* class I PI3K Dp110 has revealed a primary structure highly related to that of mammalian p110s, which act downstream of RTKs. Consistent with this, biochemical data suggest that Dp110 receives and transmits signals in a similar manner. The active enzyme exists as a heterodimer made up of the catalytic subunit, Dp110, which phosphorylates phosphoinositides on the D3 position of the inositol ring, and the SH2 domain-containing adaptor

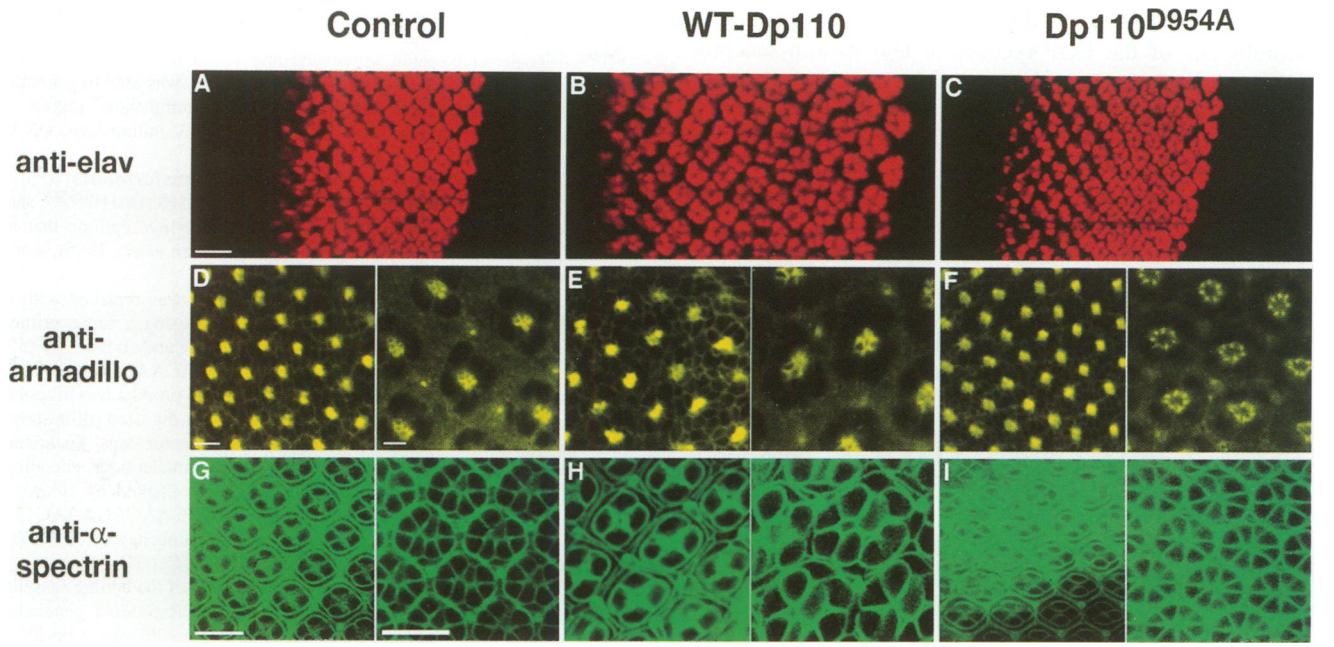

Fig. 5. Dp110 expression affects the size but not the number of cells in the eye imaginal disc. (**A–C**) Confocal images of Elav expression in the nuclei of late third instar larval eye discs; anterior is left, the equator is up. Bar 20 μm. (**D–F**) Confocal images of Armadillo immunostainings, revealing the apical surface of the photoreceptors in late third instar discs (left hand panels, anterior is left) and pupal discs (~45–50 h after puparium formation, right hand panels). Bars 5 μm. (**G–I**) Confocal images of pupal disc membranes, revealed by staining with anti-α-Spectrin. Bars 20 μm. The quartet of cone cells surrounded by pigment cells is visible in sections through apical regions of the disc (left hand panels), whereas the photoreceptor bodies are seen in more basal sections (right hand panels). Flies were of the following genotypes: (A, D and G) *GMR-GAL4/+*, (B, E and H) *GMR-GAL4, UAS-WT-Dp110/UAS-WT-Dp110*, (C, F and I) *GMR-GAL4, UAS-Dp110^{D954A}/+; UAS-Dp110^{D954A}/+*.

protein p60, which binds to the same pYXXM motifs as the mammalian enzymes.

To gain insight into the function of Dp110 *in vivo*, we have utilized the GAL4 system to overexpress either wild-type, membrane-targeted or catalytically inactive Dp110. It is possible that such an approach might result in the generation of phenotypes dependent on the cooperation of the transgene and GAL4 or that the overexpressed proteins might affect a process in which the endogenous protein is not normally involved. However, the observations that all three transgenes disrupt the same process, growth, and that catalytically inactive Dp110 has an opposing effect to that of wild-type and membrane-targeted Dp110 suggest that is not the case here. We postulated that Dp110^{D954A} would act as a dominant negative protein and compete with endogenous Dp110 for binding sites on upstream molecules such as p60 or Ras. We do not think that the Dp110^{D954A}-induced phenotypes were the result of preventing interaction of p60 or Ras with downstream effector molecules other than Dp110, since co-expression of WT-Dp110 can rescue the Dp110^{D954A} phenotypes (data not shown).

Our results implicate Dp110 in the control of imaginal disc growth, since the Dp110 transgenes affect both the size of individual cells and (in the wing disc) the overall number of cells. We are currently unable to determine how closely linked these two effects are, though it is conceivable that the differences that we see in cell number might arise as a direct consequence of the effects on cell size. An alternative possibility is that the differences in wing cell number result from a more direct effect on the rate of cell division or cell death in the developing discs. Studies in mammalian cells have implicated PI3K in the control of both processes (Valius and Kazlauskas, 1993; Yao and Cooper, 1995). Interestingly, the simultaneous disruption of two of the three genes encoding class I PI3Ks in *Dictyostelium* also affects cell growth; double knock-out strains grow slowly and have cells of reduced size (Zhou *et al.*, 1995).

Since the effect of the Dp110 transgenes on cell size might arise from alterations in biosynthesis or cytoskeletal architecture, it is noteworthy that likely downstream targets of class I PI3Ks identified in mammalian cells include P70^{S6K} (Cheatham *et al.*, 1994; Chung *et al.*, 1994) and the small GTP binding protein Rac (Wennstrom *et al.*, 1994; Hawkins *et al.*, 1995). p70^{S6K} is a serine/threonine kinase implicated in the up-regulation of translation and cell growth (Kozma and Thomas, 1994). Furthermore, studies of the class of *Drosophila* mutants termed '*Minutes*' have indicated that protein synthesis is a rate limiting step in growth and development. Heterozygous *Minute* mutations, many of which correspond to or have been genetically mapped to the vicinity of ribosomal genes, delay development and often result in flies with a reduced body size (Kongsuwan *et al.*, 1985; Lindsley and Zimm, 1992). Studies in mammalian cells have also implied a role for class I PI3Ks in the organization of the actin cytoskeleton and membrane ruffling via Rac. Thus it is possible that effects on the architecture of the cytoskeleton or membrane composition and organization contributed to the differences in cell size we observed. Furthermore, the disupted adherens junctions in Dp110^{D954A} pupal eye discs, the degeneration seen in adult eyes and the loss of wing hair polarity observed on the wing blade might all be mediated via effects on actin organization.

The phenotypes generated by ectopic Dp110 expression

suggest a possible role downstream of the *Drosophila* homologues of the EGF receceptor and insulin receptor (DER and Inr respectively). One of the mulitple phenotypes generated by mutations in DER and downstream components on the Ras/MAP kinase pathway is a decrease in wing cell size (Diaz-Benjumea and Hafen, 1994). Thus, both the Ras/MAP kinase pathway and a signal mediated by Dp110 might cooperate in the control of wing cell size. Although DER contains no YXXM motifs, the RTK substrate Dos, which is also required for correct wing cell size, contains a YXXM motif (Raabe *et al.*, 1996). Another possible upstream regulator of Dp110 is Inr, which seems to combine the function of the insulin receptor and the insulin recpetor substrate proteins IRS1 and IRS2 in mammals (Fernandez *et al.*, 1995; Chen *et al.*, 1996). Not only does Inr contain three pYXXM motifs and interact *in vitro* with the SH2 domains of mammalian p85, but flies heteroallelic for mutations in *inr* grow slowly and are significantly reduced in size (Fernandez *et al.*, 1995; Chen *et al.*, 1996). Furthermore, these growth defects arise as a result of prolonged larval instars during which the imaginal discs fail to grow to their wild-type size. Interestingly, mice deficient in IRS1 are also retarded in growth and have a reduced final body size (Araki *et al.*, 1994; Takemoto, 1994).

Although our study implies a general role for Dp110 in the control of cell growth, many aspects of endogenous Dp110 function may not have been revealed by ectopic expression. Thus it will be informative in the future to examine phenotypes generated by mutations in the gene encoding Dp110. It is of note that when we performed similar experiments to express the *Drosophila* class II and class III PI3Ks we saw no effect on growth. PI3K_59F (class III) expression gave no detectable phenotypes, whereas PI3K_68D (class II) expression produced phenotypes that implied a role in differentiation as opposed to growth. We hope that further analyses utilizing *Drosophila* as a model system will enable us to both characterize the functions of different PI3Ks during *Drosophila* development and to perform genetic screens to identify key downstream targets. The identification of such targets should help to further elucidate the mode of activation of class I PI3Ks in both *Drosophila* and mammals.

Materials and methods

Isolation and analysis of Dp110 cDNAs

All sequence data was collected using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 373A automated DNA sequencer, then analysed using the University of Wisconsin Genetics Computer Group software package. Dp110 cDNAs were isolated by screening cDNA libraries first with the 400 bp degenerate PCR product described in MacDougall *et al.* (1995) and then with subsequently isolated products. The longest cDNA (~3.5 kb), which came from a λgt10 library made from *Drosophila* eye/antennal imaginal disc cDNA (A.Cowman, unpublished), was isolated as an *Eco*RI fragment and subcloned into pBluescriptIISK (Stratagene).

To isolate sequence encompassing the initiation codon and 5′-end of Dp110, RACE–PCR was performed from the same library using a sense λgt10 primer (5′-GAGCAAGTTCAGCCTGGTAAGTC-3′) and nested antisense primers complementary to 5′-regions of the longest Dp110 cDNA isolated (primer for first PCR, 5′-ACGTGTACAGCGTATGGC-3′; primer for second PCR, 5′-GGTCGTTTGGTTACCATGGCCTGC-3′, including a unique *Nco*I site). Conditions were as described (Volinia *et al.*, 1995), except that PCRs were performed from ~10^8 p.f.u., the extension time at 72°C was reduced to 75 s and $Vent_R$ DNA polymerase (New England Biolabs) was used. Three independent 1.2 kb RACE products were isolated, *Eco*RI/*Not*I digested, subcloned into a pBluescript-derived vector, sequenced and found to be identical. The unique *Nco*I site (bp 956 of the open reading frame) was used to generate a composite cDNA made up of 294 bp of 5′ untranslated region, a 3267 bp long open reading frame and ~900 bp of 3′ untranslated DNA.

Plasmid construction and generation of transformants

pUAST transformation plasmids encoding WT-Dp110, $Dp110^{D954A}$ and Dp110-CAAX, tagged at the N-terminus with a c-myc epitope that is recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985), were constructed as follows.

First, the 3′-end of the Dp110 coding cassette was replaced with a $Vent_R$ DNA polymerase PCR product amplified using a sense primer made up of a *Kpn*I site, initiation codon, *Bam*HI site and codons 2–7 (5′-GGGGTACCACCATGGGATCCAACATGATGGACAACCGG-3′) and the *Nco*I site antisense primer described above. The product was digested with *Kpn*I and *Nco*I and subcloned into *Kpn*I/*Nco*I-digested pBluescript containing the 3.5 kb cDNA. The resulting vector was linearized with *Bam*HI and ligated to two annealed oligonucleotides encoding EQKLISEEDL (the epitope recognized by 9E10) flanked by cohesive *Bam*HI ends (sense oligonucleotide, 5′-GATCCGAACAGAAACTT-ATTTCCGAAGAGGATCTGC-3′; antisense oligonucleotide, 5′-GAT-CGCAGATCCTCTTCG GAAATAAGTTTCTGTTCG-3′).

To remove 3′ untranslated sequences, the 3′-end of the coding cassette was replaced with a $Vent_R$ DNA polymerase PCR product generated using a sense primer encompassing the unique *Bsp*106I site at bp 2857 (5′-TCACATGCATTTTGGCCAC-3′) and an antisense primer made up of an *Xba*I site, *Eco*RI site, stop codon and the last six codons (5′-GCTCTAGAGAATTCTTACTGTTTGTTGTTGTTTTTGG-3′). The product was digested with *Bsp*106I and *Xba*I, then subcloned into *Bsp*106I/*Xba*I-digested pBluescript containing the myc epitope-tagged Dp110 cassette described above.

To generate catalytically inactive Dp110 we mutated Asp954, in the Asp-Phe-Gly motif of all PI3Ks and protein kinases, to Ala. An analogous mutation has been shown to inactivate mammalian p110 (Dhand *et al.*, 1994b) and other protein kinases. A cassette encoding $Dp110^{D954A}$ was generated by cutting pBluescript-myc-Dp110 with *Bsp*106I (cuts at 2857, immediately prior to the Asp954 codon) and *Hin*dIII (cuts at 2892 and 3194), then performing a three way ligation with the vector, the 302 bp *Hin*dIII insert and two annealed oligonucleotides spanning the distance between bp 2857 and 2892 but with a point mutation converting codon 937 from GAT to GCT (sense oligonucleotide, 5′-CGCTTTTGGCCA-CATCCTGGGCCACTTCAGGAAA-3′; antisense oligonucleotide, 5′-AGCTTTTCCTTGAAGTGGCCCAGGATGTGGCCAAAAG-3′).

Membrane targeting of Dp110 was achieved by adding to the C-terminus the polybasic region and CAAX box of mammalian K-Ras (4B), which together are sufficient for the post-translational modification and membrane localization of heterologous proteins (Leevers *et al.*, 1994). An *Eco*RV site was first engineered directly upstream of the Dp110 stop codon using the PCR strategy originally used to manipulate the 3′-end of the cDNA, but with a modified antisense primer (5′-GCTCTAGAGAATTCTTACTGGATATCTTTGTTGTTGTTTTTGG-3′). The resulting plasmid was linearized with *Eco*RV and *Eco*RI and a 100 bp *Eco*RV–*Eco*RI fragment from pBluescript-Raf-CAAX (Leevers *et al.*, 1994) encoding the 15 C-terminal amino acids of c-raf and the 20 C-terminal amino acids of K-Ras, including the CAAX box, was inserted.

All PCR-derived fragments were sequenced to ensure that no unintended mutations were introduced, then the three coding cassettes were subcloned into *Kpn*I/*Xba*I-digested pUAST (Brand and Perrimon, 1993), a pCaSpeR3-based vector containing the mini-white gene in which cDNAs are expressed under the control of five GAL4 binding sites and a modified (non-heat shock-responsive) version of the hsp70 promoter. Transgenic flies were generated by injecting Qiagen-purified plasmid DNA into *yw* embryos (Basler *et al.*, 1991). Several independent transformant lines were established and analysed for each construct.

Purification of and kinase assay of mammalian and Drosophila PI3Ks

The doubly phosphorylated YXXM phosphopeptide used in this study is based on the PI3K binding sites at residues 740 and 751 of the human PDGFβ receptor: GGYMDMSKDESVDYVPML (where underlined residues represent the phosphorylated tyrosines). The peptide was coupled to Actigel-ALD Superflow resin (Sterogene Arcadia, CA) as described (1 mg peptide/ml 50% v/v slurry) (Fry *et al.*, 1992). For each binding assay, ~10^8 *Drosophila* S2 or human U937 cells were lysed for 20 min

in 1 ml lysis buffer (1% Triton X-100, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 18 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μM Na_3VO_4), then centrifuged (15 000 *g*, 20 min) and the supernatant removed and added to 5 μl phosphopeptide bead slurry and incubated for 1 h. All steps were performed at 4°C, unless indicated. The beads were washed three times in lysis buffer and twice in 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1 mM EGTA. Kinase assays were performed essentially as described (Whitman *et al.*, 1985) in 60 μl 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1 mM EGTA, 2.5 mM $MgCl_2$, 100 μM ATP containing 2.5 μCi [γ-^{32}P]ATP and ~200 μM phosphoinositide sonicated with 640 μM phosphatidylethanolamine, 600 μM phosphatidylserine, 280 μM phosphatidylcholine and 60 μM sphingomyelin. Reactions were incubated at room temperature for 30 min, then the products extracted and resolved by TLC in propan-1-ol/2 M acetic acid (65:35) containing 50 mM H_3PO_4.

Anti-Dp110 antibodies

Rabbits were immunized with the peptide CADDIQSVEVFHPLGTIE (amino acids 480–496) coupled to maleimide-activated keyhole limpet haemocyanin (Pierce). For Western blot analysis the crude antiserum was used at a dilution of 1/1000 and developed using the ECL detection system as directed (Amersham).

Drosophila stocks

Flies were raised and crossed at 25°C according to standard procedures. GAL4 was expressed in the dorsal wing pouch using the MS-1096 line (Capdevila and Guerrero, 1994), along the A/P boundary of the wing disc using a dpp-disc-GAL4 line (Staehling-Hampton and Hoffman, 1994) and behind the morphogenetic furrow of the eye disc with a GMR-GAL4 line (816), kindly provided by Matthew Freeman, in which GAL4 is under the control of five copies of the Glass binding site from the *Rhodopsin1* gene. Flies homozygous for the GMR-GAL4 insertion had rough eyes, whereas the eyes of GMR-GAL4 heterozygotes reared at 25°C were generally smooth or only slightly rough (see Figure 4A). GAL4-directed nuclear β-galactoside expression was achieved using the K17 line, kindly provided by Konrad Basler.

Adult phenotypic analysis

Wings were dehydrated in ethanol and mounted in euparal (Agar Scientific). The measurements used to generate Table I were obtained from images collected using a Kontron Progres 3008 CCD camera (Kontron Elektronic), processed in Photoshop (Adobe) and analysed using the public domain NIH Image 1.60 program (developed at the US National Institutes of Health). Scanning electron microscopy and histological sections of eyes and heads were performed as previously described (Basler *et al.*, 1991).

Immunostaining of imaginal discs

Dissected discs from wandering third instar larvae or 40–50 h pupae were fixed in 4% paraformaldehyde, 10 mM $NaIO_4$, 75 mM lysine, 30 mM $NaPO_4$ for 30 min (1 h for pupal discs) then washed in phosphate-buffered saline, 3% bovine serum albumin, 0.3% Triton X-100. Primary antibodies were used in the same solution, incubated overnight at 4°C, at the following concentrations: 9E10 mouse ascites, 1/1200; anti-α-Spectrin rabbit polyclonal 354 (Byers *et al.*, 1987), 1/500; anti-Elav rat monoclonal tissue culture supernatant (O'Neill *et al.*, 1994), 1/30; rabbit anti-β-galactosidase (5′→3′), 1/1000; mouse anti-Armadillo (Peifer *et al.*, 1994), 1/100. After washing as above, the discs were incubated for 2 h in FITC- or TRITC-conjugated secondary antibodies (Jackson Immuno-Research Laboratories), diluted 1/200–1/400, washed again and mounted in PPDA (Ashburner, 1989). Confocal images were collected using a Multiprobe 2001 confocal laser scanning microscope (Molecular Dynamics) and assembled in Photoshop.

Accession number

The EMBL nucleotide sequence accession number for the *Dp110* cDNA is Y09070.

Acknowledgements

We thank Rob Stein for anion exchange high pressure liquid chromatography, Nick Totty, Alistair Sterling, Hans Hansen and Justin Hsuan for peptide microsequencing, U.Jauch for scanning microscopy, Claude Linassier for initial cDNA characterization and Christoph Hugentobler and Patrick Faller for expert technical assistance. In addition we are grateful to Matthew Freeman for providing the GMR-GAL4 line, Daniel Branton for anti-α-Spectrin antibodies, Konrad Basler for fly stocks, the Developmental Studies Hybridoma Bank for anti-Elav and anti-Armadillo supernatants and Yamanouchi Pharmaceutical Co. Ltd for phosphopeptides. The advice of Louise Howe, Juan Riesgo and Bart Vanhaesebroeck in the preparation of this manuscript and technical advice from Damian Brunner, María Domínguez, Klaus Dücker and Juan Riesgo are also gratefully acknowledged. E.H. is supported by a grant from the Swiss National Science Foundation.

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Received on August 8, 1996; revised on September 2, 1996